Effect of labor on plasma concentrations and postpartum clearance of cell-free, pregnancy-associated, placenta-specific microRNAs

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What's already known about this topic?

- Microarray analysis has identified pregnancy-associated, placenta-specific microRNAs on C19MC.
- Circulating levels of some placental microRNAs are associated with pregnancy-associated disorders.

What does this study add?

- We show that labor increases cell-free, pregnancy-associated, placenta-specific microRNA levels in maternal plasma.
- Labor affects the postpartum clearance of plasma cell-free pregnancy-associated, placenta-specific microRNA levels 24 hours after delivery.

Abstract

Objective: This study aimed to investigate the effect of labor on plasma concentrations of cell-free, pregnancy-associated, placenta-specific microRNAs before and after delivery.

Method: In the non-labor group (32 women), cesarean section (C/S) was performed before the beginning of labor. In the labor group (32 women), C/S was performed after the beginning of labor. Plasma concentrations of cell-free, pregnancy-associated, placenta-specific microRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b) were measured by real-time quantitative RT-PCR. Each miRNA concentration was compared between the non-labor and labor groups.

Results: Before C/S, plasma concentrations of cell-free, pregnancy-associated, placenta-specific miRNAs in the labor group were significantly higher than those in the non-labor group (P=0.001 for 515-3p, P=0.002 for 517a, P=0.001 for 517c, and P=0.003 for 518b). Twenty-four hours after delivery, plasma concentrations of cell-free, pregnancy-associated, placenta-specific microRNAs in the labor group were significantly higher than those in the non-labor group (P=0.002 for 515-3p, P=0.017 for 517a, P=0.043 for 517c, and P=0.009 for 518b).

Conclusion: The presence of labor affects cell-free, pregnancy-associated, placenta-specific miRNA levels in maternal plasma. Labor also affects postpartum clearance of these microRNAs 24 hours after delivery.

Introduction

MicroRNAs (miRNAs), which function as regulators of gene expression by antisense complimentarily to specific messenger RNAs,[1-3] are expressed in tissue-specific patterns[1,2,]. Therefore, miRNAs predominantly expressed in the placenta are probably involved in placental differentiation and in the maintenance of pregnancy.[4]

The presence and detection of pregnancy-associated miRNA in maternal plasma has been previously reported.[5] Recently, we also identified pregnancy-associated, placenta-specific miRNAs (miR-515-3p, miR-517a, miR-517c, miR-518b, and miR-526b) on the chromosome 19 miRNA cluster (C19MC) region in the plasma of pregnant women.[6,7] To date, several placental miRNAs have been reported to be associated with pregnancy-associated disorders, such as preeclampsia, fetal growth restriction, and preterm delivery. Therefore, these placental miRNAs have a strong potential for use as sensitive and specific biomarkers.[8-10] A major source of cell-free placental miRNAs in maternal plasma is the villous trophoblast, which releases exosomes containing miRNAs into the maternal circulation.[11,12] Therefore, cell-free placental miRNAs have the potential for becoming novel biomarkers for prediction and

detection of pathologies in pregnancy. If cell-free miRNA levels are able to be used as predictors of pregnancy outcomes, understanding the factors that affect their circulating levels and postpartum clearance are important. However, whether labor affects circulating levels of cell-free placental miRNA in maternal plasma is unknown, although the placenta is a source of supply for cell-free pregnancy-associated, placenta-specific microRNAs.[7] Additionally, pregnancy-associated, placenta-specific miRNA concentrations are significantly decreased in maternal plasma after delivery in the placenta. However, whether labor affects the clearance of plasma cell-free placental miRNA after delivery of the placenta is also unknown.

Therefore, in this study, we aimed to investigate factors affecting circulating levels and postpartum clearance of cell-free, pregnancy-associated, placenta-specific miRNAs in maternal plasma. We investigated the association between labor and plasma concentrations of cell-free, pregnancy-associated, placenta-specific miRNAs before and after delivery.

Materials and Methods

Sample collection

All of the pregnant women in this study attended Nagasaki University Hospital or its associated hospitals. All of the samples were obtained after receiving written informed consent, and the study protocol was approved by the Institutional Review Board for Ethical, Legal, and Social Issues of Nagasaki University.

Sixty-four pregnant women at term with uncomplicated pregnancies and a singleton at 37–39 weeks' gestation, to exclude the possibility of preterm labor, were recruited. Cesarean section (C/S) was performed in all of the women. Gestational age was assessed using ultrasonography until 12 weeks of gestation. The women were divided into two groups. The labor group included 32 women who had C/S performed after the beginning of labor. In the labor group, the mean (standard deviation) time interval between the first contractions and C/S was 561.9 ± 350.4 minutes. The non-labor group included 32 women who had C/S performed before labor. The reasons for having C/S in the labor group included repeated C/S (n=14) and arrested labor (n=18), and those for having C/S in the non-labor group were repeated C/S (n=17) and malpresentation (n=15). None of the women had a history of smoking, multiple gestations, placenta previa, invasive placentation, infection, fetal anomalies or aneuploidy, fetal growth restriction, or pre-eclampsia. There were no significant differences in clinical variables, including maternal age (years), gestational age (weeks), gravidity, fetal sex, birth-weight of the newborn (g), maternal body weight (body mass index), and estimated glomerular filtration rate (eGFR) as a measure of renal function (mL/min/1.73 m^2) between the two groups (Table 1). The eGFR in all of the pregnant women was within the normal range (chronic kidney disease is defined as an eGFR <60 mL/min/1.73 m^2, and a normal to mildly reduced eGFR ranges from 60 to 89.9

mL/min/1.73 m^2).[13] Maternal blood samples (7 ml) were collected within 3 hours of C/S and 24 hours after C/S. Blood samples were placed in tubes containing EDTA. Cell-free plasma samples were prepared from maternal blood by a double centrifugation method as described previously.[7] After the first centrifugation at 3000 × g for 10 minutes, the supernatant was centrifuged at 16,000 × g for 10 min to remove blood cells. Total RNA containing small RNA molecules was extracted from 1.2 ml of maternal plasma using the *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

Real-time quantitative RT-PCR analysis of miRNAs

The pregnancy-associated, placenta-specific miRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b) were used for this study,[7] because of a limitation in sample volume. All specific primers and TaqMan probes (miR-515-3p, miR-517a, miR-517c, and miR-518b) were purchased from TaqMan MicroRNA Assays (Applied Biosystems, Warrington, UK). Real-time quantitative RT-PCR (qRT-PCR) of miRNAs in plasma samples was performed as described previously.[5-7,14,15] For each miRNA assay, we prepared a calibration curve by 10-fold serial dilution of single-stranded cDNA oligonucleotides corresponding to each miRNA sequence from 1.0×10^2 to 1.0×10^8 copies/mL. Each sample and each calibration dilution were analyzed in triplicate. Each assay could detect down to 10 RNA copies/mL.[7, 14,15] Every batch of amplifications included three water blanks as negative controls for each of the reverse transcription and PCR steps. All of the data were collected and analyzed using a LightCycler® 480 Real-Time PCR System (Roche, Pleasanton, CA, USA). There are no universally accepted internal controls in placental tissue or the maternal circulation for miRNA

analysis.[16] SnoRNAs and snRNAs, including RNU48 and RNU6B, have been suggested as reference RNAs, but have high variability.[4,10] Additionally, quantitative mRNA measurements in plasma have been recommended to be expressed as absolute concentrations.[17] Therefore, we consider that quantitative miRNA measurements may be the same as quantitative mRNA measurements in plasma. Consequently, in this study, absolute real-time qRT-PCR analysis was performed, although up- and down-regulation of miRNA expression are more adequately presented as ΔCT and 2-ΔCCT values.

Statistical analysis

Patients' backgrounds were compared by the Student's t-test and chi-square test for continuous and discrete variables, respectively, in the non-labor and labor groups. Absolute quantification data were analyzed with LightCycler® 480 software (Roche). Circulating levels of cell-free plasma concentrations of pregnancy-associated, placenta-specific miRNAs in both groups were converted into multiples of the median (MOM) in the non-labor group and adjusted for gestational age. Differences between the two groups were evaluated with the Mann–Whitney U test. Changes in cell-free plasma concentrations of placenta-specific miRNAs in the two groups before and after delivery were compared by the Wilcoxon signed-rank test. Pearson product-moment correlation coefficients were calculated between circulating levels of plasma cell-free pregnancy-associated, placenta-specific miRNAs and clinical variables (eGFR and the time interval between the first contractions and C/S). Statistical analyses were performed with SPSS software version 19 (IBM Japan, Tokyo, Japan). Significant differences were defined as P values less than 0.05.

Results

Circulating levels of plasma cell-free, pregnancy-associated, placenta-specific miRNAs in the non-labor and labor groups

Median (minimum-maximum) circulating levels of plasma cell-free pregnancy-associated, placenta-specific microRNAs in the non-labor and labor groups were 1.000 (0.298–3.031) and 1.795 (0.255–12.519) for miR-515-3p, 1.000 (0.059–7.146) and 2.730 (0.206–18.096) for miR-517a, 1.000 (0.279–12.135) and 2.028 (0.508–25.150) for miR-517c, and 1.000 (0.049–5.529) and 2.805 (0.239–22.715) for miR-518b, respectively (Figure 1). Before delivery, circulating levels of plasma cell-free, pregnancy-associated, placenta-specific microRNAs levels in the labor group were significantly higher than those in the non-labor group (Mann–Whitney U test, P=0.001 for 515-3p, P=0.002 for 517a, P=0.001 for 517c, and P=0.003 for 518b) (Figure 1).

Postpartum clearance of plasma cell-free, pregnancy-associated, placenta-specific

miRNAs 24 hours after delivery in the two groups

Maternal plasma concentrations of all four miRNAs were significantly decreased before and after delivery (Wilcoxon signed-rank test, P<0.001, Table 2, and Figures 2a-d and 3a-d).

Twenty-four hours after delivery, median (minimum-maximum) circulating levels of plasma cell-free, pregnancy-associated, placenta-specific microRNAs in the non-labor and labor groups were 1.000 (0.006–14.114) and 2.925 (0.059–10.422) for miR-515-3p, 1.000 (0.046–8.411) and 1.787 (0.222–16.427) for miR-517a, 1.000 (0.046–8.250) and 2.533 (0.271–40.387) for miR-517c, and 1.000 (0.020–6.928) and 2.565 (0.265–10.225) for miR-518b, respectively (Figure 4). Twenty-four hours after delivery, plasma concentrations of cell-free, pregnancy-associated, placenta-specific miRNAs in the labor group were significantly higher than those in the non-labor group (Mann–Whitney U test, P=0.002 for 515-3p, P=0.017 for 517a, P=0.043 for 517c, and P=0.009 for 518b) (Figure 4).

Rate of postpartum clearance of plasma cell-free, pregnancy-associated,

placenta-specific miRNAs after delivery in the two groups

The rate of postpartum clearance (decrease in concentration/h) of plasma cell-free, pregnancy-associated, placenta-specific miRNAs in the non-labor and labor groups is shown in Table 3. The postpartum clearance rate of plasma cell-free, pregnancy-associated, placenta-specific miRNAs in the labor group was significantly higher than that in the non-labor group (Mann–Whitney U test, P=0.005 for 515-3p, P=0.008 for 517a, P=0.001 for 517c, and P=0.043 for 518b). Circulating levels of plasma cell-free, pregnancy-associated, placenta-specific miRNAs before delivery and their clearance rate in the two groups were not associated with eGFR, and those in the labor group were not associated with the time interval between the first contractions and C/S (Table 4).

Discussion

This study is the first to investigate the association between labor and plasma concentrations of cell-free, pregnancy-associated, placenta-specific miRNAs before and

after delivery.

We found that, before delivery, labor increased cell-free, pregnancy-associated, placenta-specific microRNA levels in maternal plasma. Our finding is similar to previously reported data regarding cell-free fetal DNA (cff-DNA) in maternal plasma.[18] A previous study showed that external cephalic version was associated with a significant rise in fetal DNA levels.[18] The authors speculated that this increase in fetal DNA level was likely to be caused by uterine manipulation during external cephalic version. Fetal DNA in apoptotic bodies circulates in the maternal circulation. The extent of placental apoptosis may be the primary factor that affects the amount of fetal sequences released into the maternal circulation.[19,20] A source of cell-free, pregnancy-associated, placenta-specific miRNAs in maternal plasma is the villous trophoblast, which is able to release exosomes containing miRNAs into the maternal circulation.[11,12] Therefore, physical effects (uterine manipulation or uterine contraction) on the placenta appear to affect circulating cff-DNA levels and cell-free, pregnancy-associated, placenta-specific miRNA levels in maternal plasma. The placenta-specific miRNAs (miR-515-3p, miR-517a, miR-517c, and miR-518b)

analyzed in this study were located on the C19MC region, which is imprinted in the placenta, with expression from the paternally inherited chromosome.[21] MicroRNAs are non-protein coding small RNAs (21–25 nucleotides) that function as regulators of gene expression by antisense complimentarily to specific messenger RNAs.[1-3] Therefore, alterations in plasma concentrations of placental miRNA may change the environment *in utero*.[22,23] Compared with cff-DNAs, which are fragmented to less than 100 bp,[24] the advantage of cell-free, pregnancy-associated, placenta-specific miRNAs in maternal plasma is that they are a sex-independent marker and a smaller molecule for easier communication between the fetus and mother.[5-7] Therefore, placental miRNAs may be important molecules for clarifying the mechanism of labor.

With regard to the effect of labor on postpartum clearance of pregnancy-associated, placenta-specific microRNA levels in maternal plasma, we found that maternal plasma concentrations of all four miRNAs were significantly decreased after delivery of the placenta. The presence of labor increased plasma concentrations of all four cell-free pregnancy-associated, placenta-specific miRNAs in women 24 hours after delivery. Our finding is similar to previously reported data on cff-DNA in maternal plasma.[25] A

previous study also showed that the presence of labor increases the rate of detectable cff-DNA in maternal plasma on days 1–2 postpartum.[25] Therefore, the process of labor may cause an increase in the release of cff-DNA from the trophoblast, and this is possibly mediated by contraction-induced hypoxia at the maternal-placental interface and physical effects.[18,25] Repetitive uterine contractions and subsequent disturbance to myometrial perfusion may cause hypoxia at the placental interface, leading to increased cff DNA and cell-free, pregnancy-associated, placenta-specific miRNA release into maternal plasma during labor.[25]

Furthermore, the rate of postpartum clearance of plasma cell-free, pregnancy-associated, placenta-specific miRNAs in the labor group was significantly higher than that in the non-labor group. This finding suggests that labor affects circulating levels before delivery and the postpartum clearance rate of plasma cell-free placental miRNAs. However, the time interval between the first contractions and C/S was not associated with plasma concentrations of cell-free, pregnancy-associated, placenta-specific miRNAs before C/S or the clearance rate of cell-free, pregnancy-associated, placenta-specific miRNAs. Our findings are similar to previously

reported data of cff-DNA and cell-free placental mRNA in maternal plasma.[26] However, the mechanism regarding the lack of association between the duration of labor and the clearance rate of plasma cell-free placental nucleic acids is still unknown. In a previous study, clearance of plasma cell-free placental mRNA appeared to be delayed, with levels remaining high at 24 hours post-delivery in cases of preeclampsia that showed both pregnancy-induced hypertension and proteinuria.[26] However, in cases of uncomplicated renal function, we found that there was no relation between eGFR within the normal range and the clearance rate of plasma cell-free, pregnancy-associated, placenta-specific miRNAs. Therefore, abnormality of renal function appears to affect circulating levels of plasma cell-free, pregnancy-associated, placenta-specific miRNAs before delivery, as well as their postpartum clearance.

In conclusion, our study shows that labor is an important factor for increasing cell-free, pregnancy-associated, placenta-specific miRNA levels in maternal plasma. Additionally, labor affects the postpartum clearance of these miRNAs 24 hours after delivery. Similar to the physiological mechanism of cff-DNA and cell-free placental mRNA,[25-27] elevation of cell-free, pregnancy-associated, placenta-specific miRNAs in

maternal plasma may be a molecular marker of considerable uterine activity and fetomaternal hemorrhage. Further research on the normal physiology of cell-free, pregnancy associated, placenta-specific miRNAs in maternal plasma is important for establishing quantitative applications of these molecules for screening for pregnancy-associated diseases.

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Table 1. Clinical characteristics of pregnant women.

Characteristics	Non-labor group (n=32)	Labor group (n=32)	P value
Maternal age (years)	32.0 (4.8)[a]	31.1 (4.6) [a]	NS
Gestational age at sampling (weeks)	38.2 (1.6) [a]	38.1 (0.9) [a]	NS
Parity			NS
Nulliparous	12	18	
Primiparous	13	9	
Multiparous	7	5	
Placental weight (g)	561.3 (92.8) [a]	562.5 (109.9) [a]	NS
Fetal birth weight (g)	2962.3 (379.1) [a]	3034.8 (354.7) [a]	NS
BMI (kg/m2)	21.2 (2.3) [a]	20.6 (2.5) [a]	NS
eGFR (mL/min/1.73 m^2)	107.31 (14.62) [a]	117.67 (22.56) [a]	NS
Time interval (min) [b]	0	561.9 (350.4) [a]	
Newborn sex			NS
Male	12	17	
Female	20	15	

[a]Values are expressed as the mean and (SD). Significant differences between groups were analyzed by the Student's t-test or chi-square test. A P value <0.05 was considered significant.

[b]Time interval between the first contractions and cesarean section.

BMI, body mass index; NS, not significant; eGFR, estimated glomerular filtration rate.

Table 2. Circulating levels of pregnancy-associated, placenta-specific miRNAs in maternal plasma from uncomplicated pregnant women with or without labor before and after delivery.

microRNA	Non-labor group (n=32)		P value	Labor group (n=32)		P value
	Before delivery (copies/mL)	After delivery (copies/mL)		Before delivery (copies/mL)	After delivery (copies/mL)	
miR-515-3p	5126.5 (1528.2–15541.1)	482.6 (27.9–6810.8)	<0.001	9200.7 (1306.3–64178.5)	1411.6 (28.5–5029.0)	<0.001
miR-517a	33596.6 (1985.2–240108.3)	562.6 (25.9–4732.4)	<0.001	91715.8 (6912.7–607950.7)	1005.2 (124.9–9242.4)	<0.001
miR-517c	38135.0 (10653.0–462770.1)	926.2 (42.2–7641.2)	<0.001	77325.2 (19366.7–959079.2)	2345.8 (251.0–37404.3)	<0.001
miR-518b	4269.4 (211. 9–23608.3)	626.0 (12.3–4337.0)	<0.001	11976.5 (1019.1– 96979.0)	1605.7 (165.9– 6400.6)	<0.001

Circulating levels are expressed as median (minimum-maximum) copies/mL. Changes in cell-free plasma concentrations of placenta-specific miRNAs in the two groups before and after delivery were compared by the Wilcoxon signed-rank test. A P value <0.05 was considered significant.

Table 3. Rate of postpartum clearance of pregnancy-associated, placenta-specific miRNAs in the non-labor and labor groups.

	Non-labor group (n=32) (copies/mL/h)	Labor group (n=32) (copies/mL/h)	P value
miR-515-3p	200.8 (22.6–2153.8)	295.8 (50.3–2555.3)	0.005
miR-517a	1305.8 (75.0–46,159.4)	3768.6 (273.7–106,836.1)	0.008
miR-517c	1471.3 (330.4–19,186.2)	3119.2 (691.7–39,836.3)	0.001
miR-518b	119.6 (0.2–4294.4)	355.0 (7.1–10,658.0)	0.043

The rate of postpartum clearance of cell-free miRNAs is expressed as median (minimum-maximum) copies/mL/h. A decrease in plasma concentrations of cell-free, pregnancy-associated, placenta-specific miRNAs after delivery per hour in the two groups was compared by the Mann–Whitney U-test. A P value <0.05 was considered significant.

Table 4. Summary of correlation coefficient analysis between plasma concentrations of cell-free, pregnancy-associated, placenta-specific miRNAs and clinical variables.

Groups	Pregnancy-associated, placenta-specific miRNAs	Plasma concentrations of cell-free miRNA in maternal plasma	Clinical variables			
			eGFR (mL/min/1.73 m^2)		Time interval* (min)	
			r value	P value	r value	P value
Labor group	miR-515-3p	Concentration before delivery	-0.380	0.249	0.070	0.703
		Rate of postpartum clearance	-0.393	0.232	0.077	0.674
	miR-517a	Concentration before delivery	-0.154	0.651	-0.133	0.468
		Rate of postpartum clearance	-0.155	0.650	-0.133	0.469
	miR-517c	Concentration before delivery	-0.245	0.469	-0.074	0.685
		Rate of postpartum clearance	-0.250	0.458	-0.070	0.703
	miR-518b	Concentration before delivery	-0.137	0.687	-0.029	0.874
		Rate of postpartum clearance	-0.131	0.700	0.075	0.685
Non-labor group	miR-515-3p	Concentration before delivery	-0.126	0.586		
		Rate of postpartum clearance	-0.154	0.506		
	miR-517a	Concentration before delivery	-0.006	0.980		
		Rate of postpartum clearance	-0.003	0.991		
	miR-517c	Concentration before delivery	-0.048	0.836		
		Rate of postpartum clearance	-0.051	0.827		
	miR-518b	Concentration before delivery	-0.235	0.304		
		Rate of postpartum clearance	-0.232	0.313		

A P value <0.05 was considered significant.
*Time interval between the first contractions and cesarean section.
eGFR, estimated glomerular filtration rate.

Figure Legends

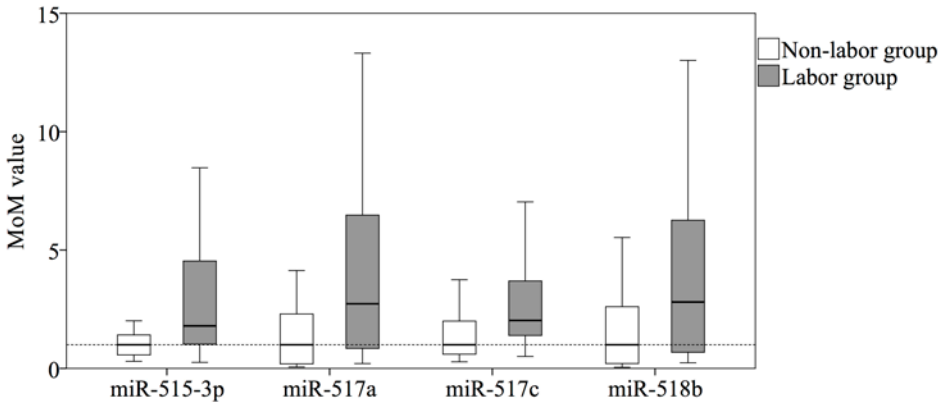

Figure 1. Plasma concentrations of pregnancy-associated, placenta-specific miRNAs within 3 hours of delivery in the non-labor and labor groups.

Plasma concentrations are expressed as multiples of the median (MoM) values. White bars indicate data from the non-labor group (n=32) and gray bars indicate data from the labor group (n=32). Differences between the two groups were evaluated with the Mann–Whitney U test. Significant differences were defined as P values less than 0.05.

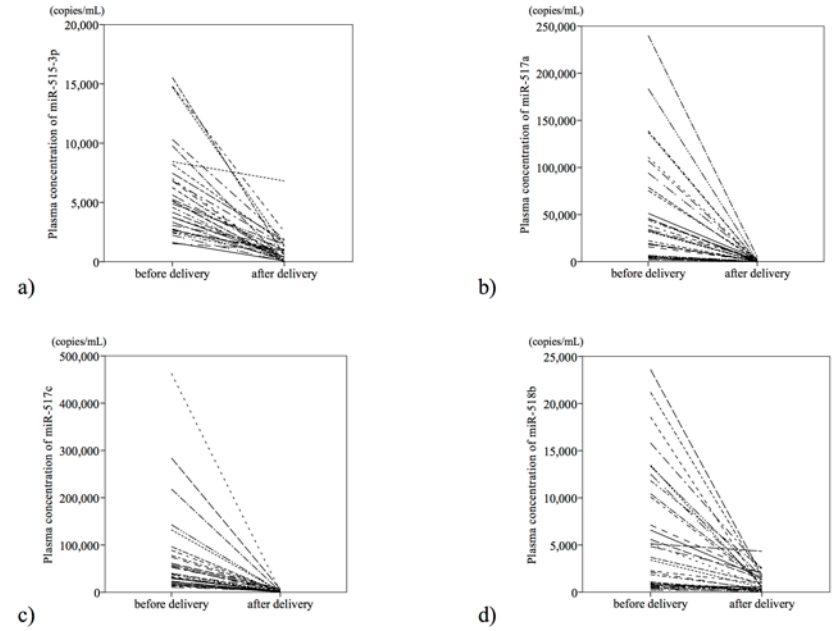

Figure 2.

Figure 2. Changes in plasma concentrations of cell-free, placenta-specific miRNAs in the non-labor group before and after delivery.

(a) miR-515-3p, (b) miR-517a, (c) miR-517c, and (d) miR-518b. Circulating levels of miRNAs in maternal plasma are expressed as copies/mL. Plasma levels of all four miRNAs were significantly decreased after delivery (Wilcoxon signed-rank test, $P<0.001$).

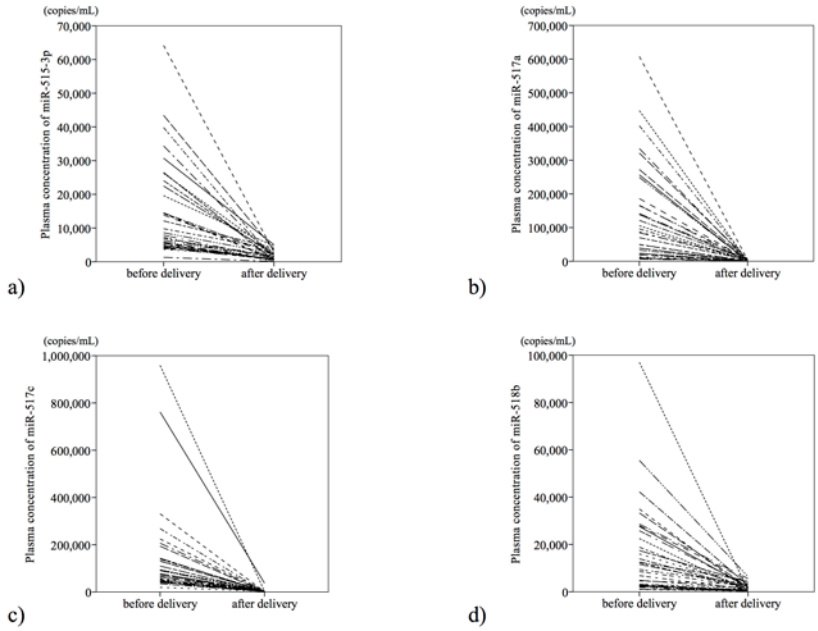

Figure 3.

Figure 3. Changes in plasma concentrations of cell-free, placenta-specific miRNAs in the labor group before and after delivery.

(a) miR-515-3p, (b) miR-517a, (c) miR-517c, and (d) miR-518b. Circulating levels of miRNAs in maternal plasma are expressed as copies/mL. The plasma levels of all four miRNAs were significantly decreased after delivery (Wilcoxon signed-rank test, $P<0.001$).

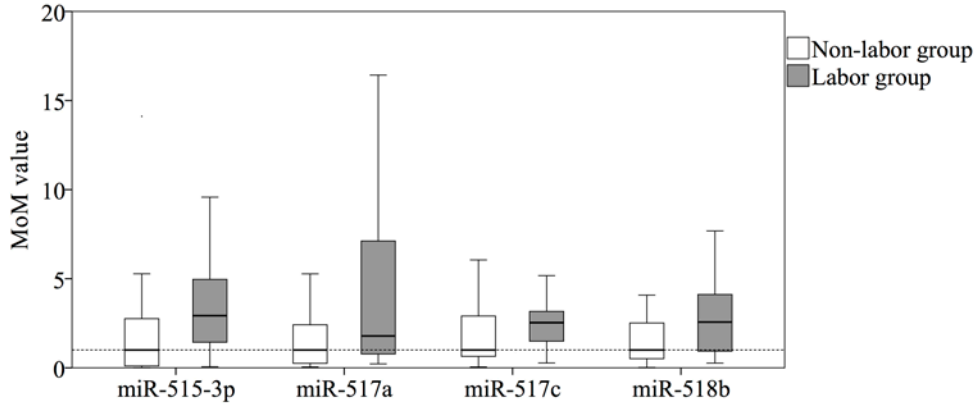

Figure 4. Plasma concentrations of pregnancy-associated, placenta-specific miRNAs 24 hours after delivery in the non-labor and labor groups.

Plasma concentrations are expressed as multiple of median (MoM) values. White bars indicate data from the non-labor group (n=32) and gray bars indicate data from the labor group (n=32). Differences between the two groups were evaluated with the Mann–Whitney U test. Significant differences were defined as P values less than 0.05.